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Effects of insulin-degrading enzyme on the proliferation of swine Sertoli cells

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ABSTRACT

Sertoli cells, the only somatic cells in testis seminiferous tubules, provide a supporting microenvironment for male germ cells and play essential roles in spermatogenesis. The insulin-degrading enzyme (*IDE*), a ubiquitous zinc peptidase of the inverzincin family, plays crucial role in sperm production, as IDE-knockout mice presented decreased testis weight and impaired sperm viability and morphology. However, whether and how *IDE* affects swine Sertoli cell proliferation remains unclear. Thus, in the present study, we aimed to evaluate the effects of *IDE* on the proliferation of swine Sertoli cells, as well as its underlying molecular mechanism. After knocking down *IDE* expression with small interfering RNA transfection, we analyzed the proliferation of swine Sertoli cells as well as the expression of related regulatory factors (*WT1*, *ERK*, and *AKT*). The results showed that *IDE* knockdown promoted swine Sertoli cell proliferation and increased *WT1* expression, possibly through activating ERK and AKT. Overall, our findings suggest that *IDE* may be involved in male reproduction by regulating Sertoli cell proliferation, which provides new information to better understand the regulatory mechanisms of swine Sertoli cells and improve the reproductive traits of male pigs.

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Introduction

Sertoli cells, as the only somatic cells in the testis seminiferous tubules, play crucial roles in testis development and spermatogenesis. Mammalian spermatogenesis is dependent on Sertoli cells because of their structural, immune, and nutritional support to germ cells at different developing stages, including spermatogonia, spermatocytes, spermatids, and spermatozoa (1, 2). Numerous factors play important regulatory roles in Sertoli cell proliferation (3); however, the underlying molecular mechanisms of the regulation remain unclear. Understanding the molecular mechanisms underlying swine Sertoli cell proliferation is necessary to better understand the process of spermatogenesis and improve male reproductive traits in pigs.

WT1 transcription factor (*WT1*) is specifically expressed in Sertoli cells and essential for testis development, as the specific deletion of *WT1* in mouse Sertoli cells can cause testicular cord disruption and testis dysgenesis (4). Moreover, *WT1* deficiency in neonatal testes resulted in the accumulation of undifferentiated spermatogonia and disrupted the meiotic progression of spermatocytes (5). *WT1* depletion in postnatal Sertoli cells led to increased germ cell apoptosis and impaired fertility (6). Similarly, *WT1* knockout in adult testes causes massive germ cell death in the seminiferous tubules (7). These findings demonstrate that *WT1* functions as a crucial regulator of spermatogenesis in Sertoli cells. In addition, it is also reported that the ERK1/2 and PI3K/AKT pathways are main signal transduction pathways involved in Sertoli cell proliferation (3). Among these pathwas, AKT activation is positively involved in swine Sertoli cell proliferation (8) and *WT1* knockdown can reduce AKT activity (9). ERKs are central regulators of multiple cellular processes. The phosphorylated of Thr202/Tyr204 residues in ERK1 and Thr185/Tyr187 residues in ERK2 mediates protein activation (10). Indeed, ERK signaling primarily controls the cell cycle, as evidenced by the arrest of fibroblast proliferation when ERK1 and ERK2 lost their function (11). This pathway is also associated with bovine and chicken primary Sertoli cell proliferation (12, 13).

The insulin-degrading enzyme (*IDE*) is a ubiquitous zinc peptidase of the inverzincin family and exhibits a highly conserved primary sequence in all species (14). Initially, the biological role of *IDE* was associated with insulin degradation (15, 16). Dysfunction of *IDE* causes glucose intolerance, which is correlated with the pathogenesis of type 2 diabetes (17-19). Besides insulin, *IDE* can degrade amylin and glucagon, suggesting a regulatory role in carbohydrate metabolism (14, 20, 21). Moreover, *IDE* as a major endogenous amyloid beta-degrading enzyme mediates the clearance of amyloid beta and is negatively associated with Alzheimer's disease (17, 22). These findings suggest that *IDE* serves as an important regulatory factor in the association between type 2 diabetes and Alzheimer's disease (23, 24). In addition, *IDE* was found in both human cancer and normal tissues, leading to the investigation of whether *IDE* can serve as a diagnostic or

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prognostic marker for cancer progression (25, 26). Furthermore, reduced *IDE* expression sustained myoblast proliferation, but delayed myogenic differentiation in mice (27). Similarly, reducing *IDE* in porcine skeletal muscle stem cells promoted proliferation and mitigated cell apoptosis (28). Another study reported that IDE-knockout mice exhibited testicular morphological changes and impaired sperm quality. These mice had decreased testis weight and seminiferous tubule diameter with impaired sperm quality, likely due to decreased sperm viability and impaired sperm morphology (29). These findings demonstrated that *IDE* plays an important role in male reproduction.

Given the importance of understanding the molecular mechanisms behind Sertoli cell proliferation and the reproductive function of *IDE* in males, the role of *IDE* in swine Sertoli cell proliferation was the focus of this study. *IDE* knockdown mediated by small interfering RNA transfection was performed in swine Sertoli cells to identify its role during proliferation, followed by examining *WT1* expression and ERK/AKT activities. The results showed that *IDE* knockdown promoted the proliferation of swine Sertoli cells and increased *WT1* expression, which might be associated with ERK/AKT activation. Our findings provide new information about the regulatory mechanisms in swine Sertoli cells, which is beneficial for improving reproductive traits in male pigs.

Materials and Methods

Cell culture

Swine Sertoli cells used in this study were purchased from Boster (CX0300, Wuhan, China). They were cultured in high-glucose Dullbecco's modified Eagle's medium (HyClone, UT, USA) supplemented with 10% fetal bovine serum (Gibco, TX, USA) at 37 °C in a humidified atmosphere with 5% CO_2.

Small interfering RNA transfection

IDE small interfering RNA (*IDE* siRNA; targeting sequence: GGAATGAAGTTCACAATAA) and negative control (NC) siRNA were designed and synthesized by RiboBio (Guangzhou, China). Briefly, swine Sertoli cells were seeded in a cell culture plate at a density of 5000 cells/cm^2 and incubated overnight. Then 50 nM *IDE* siRNA or NC siRNA was transfected into the cells using Lipofectamine RNAiMAX reagent (Invitrogen, MA, USA) following the manufacturer's instructions. The swine Sertoli cells were collected for further analysis after 48 h of transfection.

RT-qPCR analysis

Total RNA was extracted from swine Sertoli cells using TRIzol reagent (Invitrogen). For cDNA synthesis, 0.5 μg RNA was reverse transcribed using the PrimeScript™ RT reagent kit with a gDNA eraser (TaKaRa, Shiga, Japan). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in a 20 μL reaction volume that included 10 μL SYBR Select Master Mix (2×; TaKaRa), 1 μL cDNA, 0.2 μM each forward and reverse primers, 0.4 μL ROX reference dye II, and double-distilled H_2O. The ABI 7500 Fast Real-Time PCR system (Applied Biosystems, MA, USA) was used to run the reactions. Relative mRNA expression was determined by normalizing the expression of the target gene against that of *GAPDH* using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used for RT-qPCR are listed in Table 1. Relative mRNA expression results are presented as fold-change from the control.

Western blotting analysis

Total protein was extracted from swine Sertoli cells using a protein extraction reagent supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, MA, USA). Denatured protein samples were separated using 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membranes (Millipore, MA, USA). Membranes were blocked with 5% non-fat milk for 1 h at 25 °C and incubated with primary antibodies overnight at 4 °C. The primary antibodies included IDE (1:1000; ab33216, Abcam, Cambridge, UK), WT1 (1:1000; ab89901, Abcam), p-ERK1/2 (1:1000; #4370, Cell Signaling Technology, MA, USA), ERK1/2 (1:2000; 67170, Proteintech, Wuhan, China), p-AKT (1:1000; #9271, Cell Signaling Technology), AKT (1:2000; 60203, Proteintech), and GAPDH (1:1000; #2118, Cell Signaling Technology). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The blots were developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and protein bands were visualized on a Tanon-5200 Chemiluminescent Imaging System. The bands were quantified using integrated density, which was calculated using ImageJ software. The protein expression level was normalized to that of endogenous GAPDH.

Cell proliferation analysis

Swine Sertoli cell proliferation was evaluated using Cell Counting Kit 8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Brie-

Table 1. Primers used for RT-qPCR to detect gene relative expression.

Gene name		Primer sequences
GAPDH	F	AGGGCTGCTTTTAACTCTGGCAA
	R	GATGGTGATGGCCTTTCCATTG
IDE	F	AAGCAGGCCGCATTAGGAAT
	R	GCTCTGCATGTCTGTCTGGT
CCNA2	F	CTAACATTGCAGCAGACGGC
	R	CTTAAGAGGCGCAACCCGT
CDK2	F	TTTGCTGAGATGGTGACCCG
	R	AAAATCTTGCCTGGCCCACT
BCL2	F	GAGGCTGGGATGCCTTTGTG
	R	CCACAGCTTCTTTGCATGGT
BAX	F	GCCCTTTTGCTTCAGGGTTTC
	R	TGCCGTCAGCAAACATTTCG
P53	F	GCTTTGAGGTGCGTGTTTGT
	R	TTCAGCTCCAAGGCGTCATT
WT1	F	TGAGCGAAGGTTTTCTCGTT
	R	GCTGAAGGGCTTTTCACTTG
ERK1	F	GCTACACGCAGCTGCAATAC
	R	CATCCCTCATGGCTTCCAGG
ERK2	F	TGGCAGATATGCTCTCCAAC
	R	AGTCAGCATTTGGGAACAGC
AKT3	F	GGCCTTGGGGTTGTCATGTA
	R	GTCCTCCACCAAGGCGTTTA

fly, swine Sertoli cells were seeded at a density of 2000 cells per well in a 96-well plate and cultured overnight. After the cells were transfected with siRNAs for 48 h, 10 μL CCK-8 solution was added to the medium. Then, the cells were incubated at 37 °C for 1 h and the absorbance was measured with a microplate reader (SpectraMax M5; Molecular Devices, CA, USA) at a wavelength of 450 nm. Cell viability (%) was calculated using the following equation: (Absorbance of IDEsi group - Absorbance of blank)/ (Absorbance of NC group - Absorbance of blank) × 100.

Immunofluorescence staining

Swine Sertoli cells were washed with DPBS and fixed in 4% PFA, followed by permeation in 0.1% Triton-X100. After 1 h blocking with 3% BSA, the cells were incubated with WT1 primary antibody ((1:50; ab89901, Abcam)) overnight at 4 °C. With three times of washing, these cells were incubated with secondary antibody (1:1000; A32740, Invitrogen, MA, USA) at room temperature in dark for 1 h. Thereafter, the cells were washed and the nuclei were stained with Hoechst 33342 (C1025, Beyotime, Shanghai, China) in dark for 5 min. Finally, the cells were observed under a fluorescence microscope (Leica DMI 6000B).

Statistical analysis

Data are presented as the mean ± SEM from three independent experiments. Statistical analysis of difference was calculated using the Student's t-test. Statistical significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Results

Downregulation of *IDE* enhances swine Sertoli cell proliferation

To investigate the role of *IDE* in swine Sertoli cell proliferation (morphology was shown in Figure 1A), *IDE* siRNA was used to inhibit *IDE* expression. After swine Sertoli cells were transfected with *IDE* siRNA for 48 h, *IDE* expression was evaluated at both the mRNA and protein levels. The results showed that the expression levels of *IDE* mRNA were significantly decreased in the *IDE* knockdown group compared to those of the control group (Figure 1B; *** $p < 0.001$). Similarly, Western blotting analysis showed that IDE protein levels were dramatically decreased after *IDE* knockdown (Figure 1C and D; *** $p < 0.001$). Next, the CCK-8 assay was used to analyze swine Sertoli cell proliferation with and without *IDE* knockdown. After 48 h of siRNA transfection, swine Sertoli cells in the *IDE* knockdown group showed enhanced cell viability compared to that in the control group (Figure 1E; ** $p < 0.01$). These data demonstrated that *IDE* knockdown promoted swine Sertoli cell proliferation.

IDE knockdown increases the expression levels of cell cycle-regulating factors but decreases those of apoptosis-inducing factors in swine Sertoli cells

Given the enhanced swine Sertoli cell proliferation after *IDE* downregulation, the expression levels of the cell cycle-related factors, *CCNA2* and *CDK2*, and apoptosis-related factors, *BCL2*, *BAX*, and *P53*, were evaluated in Sertoli cells with NC siRNA and *IDE* siRNA transfection. The RT-qPCR results revealed that *IDE* knockdown dramatically increased the expression levels of *CCNA2*, *CDK2*, and *BCL2*, and significantly decreased expression levels of *BAX* and *P53* when compared with control group (Figure 2 A and B, * $p < 0.05$; ** $p < 0.01$). These results indicated that *IDE* knockdown enhanced swine Sertoli cell proliferation by promoting cell cycle progression and mitigating cell apoptosis.

IDE negatively regulates *WT1* expression in swine Sertoli cells

WT1, a marker of Sertoli cells, plays an important role in maintaining Sertoli cell lineage and spermatogenesis. Therefore, we examined the expression of *WT1* to investigate the effect of *IDE* downregulation. As expected, *WT1*

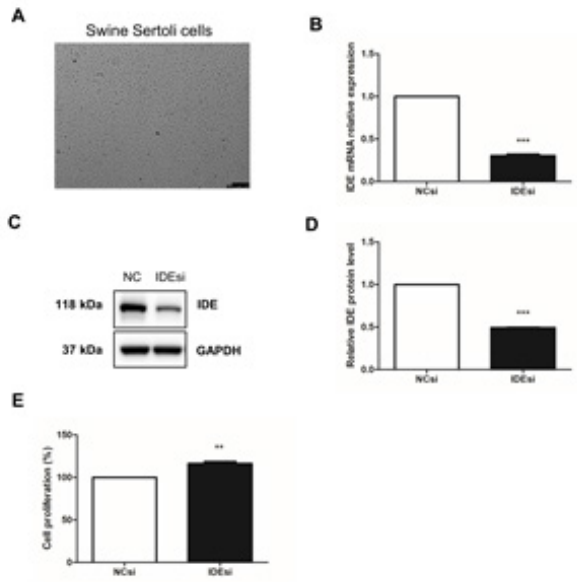

Figure 1. Downregulation of *IDE* enhances swine Sertoli cell proliferation. (A) Morphology of swine Sertoli cells were shown under microscope (scale bar = 100 μM). (B) *IDE* mRNA expression levels after 48 h siRNA transfection were examined by RT-qPCR. (C–D) IDE protein levels after 48 h siRNA transfection were determined using Western blotting and quantified via integrated density analysis using ImageJ software. (E) Cell proliferation analysis after siRNA transfection for 48 h was determined using the CCK-8 assay. Data are shown as mean ± SEM from three independent experiments. ** $p < 0.01$ and *** $p < 0.001$.

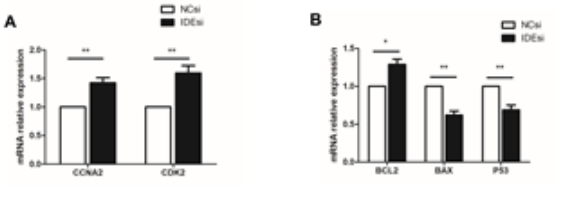

Figure 2. *IDE* knockdown increases the expression of cell cycle-regulating factors and decreases that of apoptosis-inducing factors in swine Sertoli cells. Relative mRNA expression levels of (A) *CCNA2* and *CDK2* and (B) *BCL2*, *BAX*, and *P53* after siRNA transfection for 48 h were detected using RT-qPCR. Data are shown as mean ± SEM from three independent experiments. * $p < 0.05$ and ** $p < 0.01$.

was positively expressed in swine Sertoli cells (Figure 3A). RT-qPCR results showed that IDE knockdown significantly increased *WT1* mRNA levels compared with those in the control group (Figure 3B; * $p < 0.05$). Similarly, Western blotting analysis revealed that WT1 protein levels were dramatically higher in IDE-knockdown swine Sertoli cells compared with those in the control group (Figure 3C and D; * $p < 0.05$). These results suggested that *IDE* negatively regulated *WT1* expression in swine Sertoli cells.

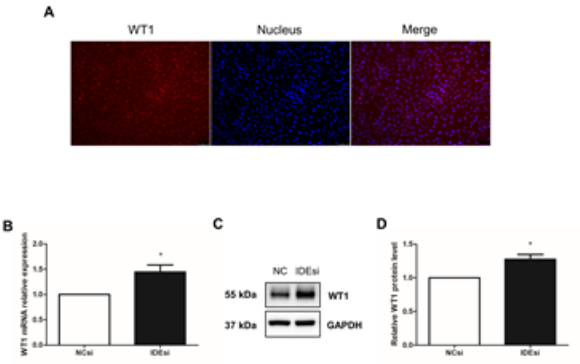

Figure 3. *IDE* negatively regulates *WT1* expression in swine Sertoli cells. (A) WT1 was positively expressed in swine Sertoli cells determined by immunofluoresence staining (scale bar =50 μM). (B) *WT1* mRNA expression levels after siRNA transfection for 48 h were analyzed using RT-qPCR. (C–D) WT1 protein levels after siRNA transfection for 48 h were analyzed using Western blotting and ImageJ software by caculating the integrated density of the protein bands. Data are shown as mean ± SEM from three independent experiments. * $p < 0.05$.

Downregulated *IDE* expression enhances ERK activity in swine Sertoli cells

To elucidate the possible mechanism of *IDE* regulation during swine Sertoli cell proliferation, *ERK* expression and its phosphorylation level (indicating its activation) were examined. When the Sertoli cells were transfected with *IDE* siRNA for 48 h, both *ERK1/2* mRNA expression and phosphorylated ERK1/2 (p-ERK1/2)/ ERK ratio were increased significantly, compared with those of the control group (Figure 4A–C; * $p < 0.05$). These findings suggested that downregulating *IDE* enhanced ERK activity in swine Sertoli cells.

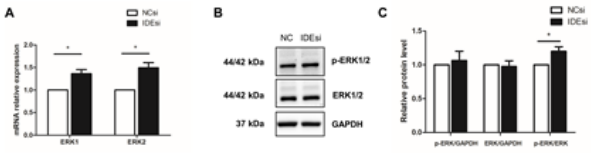

Figure 4. Downregulation of *IDE* enhances ERK activity in swine Sertoli cells. (A) Relative mRNA expression levels of *ERK1* and *ERK2* after 48 h siRNA transfection were determined by RT-qPCR. (B) ERK1/2 protein and phosphorylation levels after 48 h siRNA transfection were examined using Western blotting. (C) p-ERK, ERK, and p-ERK/ ERK ratio of relative protein level were analyzed by measuring the integrated density of bands using Image J software. Data are shown as mean ± SEM from three independent experiments. * $p < 0.05$.

Downregulated *IDE* expression enhances AKT activity in swine Sertoli cells

In addtion to ERK activity, AKT activation is also positively involved in swine Sertoli cell proliferation. Therefore, we investigated whether *IDE* expression could affect AKT activity in swine Sertoli cells. To this end, the expression of *AKT3* (an AKT isoform abundant in the testis and brain) and phosphorylated AKT (p-AKT) were further identified. The RT-qPCR and Western blotting analyses demonstrated that *AKT3* mRNA and p-AKT/AKT ratio were significantly increased in the IDE-downregulated group compared to those in the control group (Figure 5A–C; * $p < 0.05$, *** $p < 0.001$). These results suggested that *IDE* downregulation enhanced AKT activity in swine Sertoli cells.

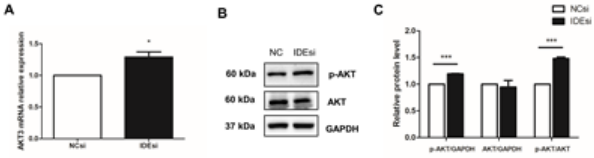

Figure 5. Downregulation of *IDE* enhances AKT activity in swine Sertoli cells. (A) Relative mRNA expression levels of *AKT3* after 48 h siRNA transfection were measured by RT-qPCR. (B) AKT protein and phosphorylation levels after 48 h siRNA transfection were determined by Western blotting. (C) p-AKT, AKT, and p-AKT/AKT ratio of relative protein levels were caculated by the integrated density of bands using ImageJ software. Data are shown as mean ± SEM from three independent experiments. * $p < 0.05$ and *** $p < 0.001$.

Discussion

The proliferation of testicular Sertoli cells affects spermatogenenic function in the testes, determining the final volume of the testes and sperm production (1, 2), which is closely correlated to the reproductive health of humans and the reproductive traits of the pigs. *IDE* knockout in mice led to decreased testis weight, reduced seminiferous tubule diameter, and impaired sperm quality with decreased sperm viability and morphology (29), demonstrating that *IDE* plays an important role in determining the reproductive potential of males. In the current study, we aimed to identify the effects of *IDE* on the proliferation of swine Sertoli cells and its underlying molecular mechanisms. Our results showed that *IDE* knockdown promoted the proliferation of swine Sertoli cells, possibly through activating the ERK and AKT signaling pathways.

We found that siRNA-mediated *IDE* knockdown enhanced the proliferation of swine Sertoli cells, followed by increased expression levels of proliferation-related genes (*CCNA2* and *CKD2*). Currently, the role of *IDE* in controlling cell growth and proliferation is believed to be complex because *IDE* is considered a tumor suppressor or novel oncogene (14). Although *IDE* acts as a positive regulator in a variety of human tumor cell lines or tissues, it is also expressed in normal tissues (25, 26). Tundo et al. reported that *IDE* was overexpressed in tumors of the central nervous system, while silencing *IDE* inhibited cell proliferation and triggered the death of neuroblastoma cells (30). However, Epting et al. and our previous work revealed that *IDE* negatively regulated the proliferation of

mouse myoblasts and porcine skeletal muscle stem cells, respectively (27, 28). In addition, there are structural similarities between the retinoblastoma tumor suppressor protein and *IDE*, as well as between *IDE* and *CDK* inhibitors such as p16 and p21 (31). Taking these fingdings together, the effect of *IDE* on the proliferation of swine Sertoli cells is similar to that of muscle cells, suggesting that *IDE* negatively regulates the proliferation of swine Sertoli cells and is correlated with expression changes in cell-cycle regulators.

We next examined the effect of *IDE* on the expression of *WT1*, a marker for Sertoli cells and an important factor for spermatogenesis (4). The results revealed that both *WT1* mRNA and protein expression levels increased after *IDE* knockdown in swine Sertoli cells, which highlights an intriguing phenomenon regarding the actual role of *IDE* in spermatogenesis. As known in many reports, the spermatogenesis processes such as spermatogonia differentiation, and spermatocyte meiosis were impaired and massive germ cells underwent death in WT1-deficient testes (5, 7); the WT1-deleted Sertoli cells led to increased germ cell apoptosis and impaired fertility (6). These findings demonstrate the negative effects of loss-of-funciton *WT1* on spermatogenesis. However, it is noteworthy that *WT1* overexpression in Leydig cells led to the upregulation of Sertoli cell-specific genes and downregulation of steroidogenic genes (32). Therefore, we hypothesize that increased *WT1* expression occurs in Sertoli cells as well as in other testicular cells, such as Leydig cells, and can disrupt the function of Leydig cells, consequently imparing the testicular environment required for normal spermatogenesis. This hypothesis will be further explored in the future work.

To further identify the possible molecular mechanisms of *IDE* in regulating swine Sertoli cell proliferation, we examined the activity of ERK and AKT. It is reported that ERK activity is associated with the proliferation and apoptosis of bovine primary Sertoli cells (12) and the melatonin-induced cell proliferation of chicken Sertoli cells (13). We investigated whether downregulation of *IDE* would affect ERK1/2 by measuring ERK1/2 phosphorylation. As expected, both the *ERK1/2* mRNA expression and the phosphorylated ERK (p-ERK)/ERK ratio increased after *IDE* knockdown, suggesting the *IDE*-mediated cell proliferation is regulated through ERK activation in swine Sertoli cells. The AKT and ERK pathways are both important cellular signaling cascades, with large functional overlap in cell proliferation and apoptosis, indicating their close cross-talk relationship (33). AKT activity is known to be positively involved in the proliferation of swine Sertoli cells (8). Therefore, we determined *AKT3* expression and the phosphorylated AKT (p-AKT)/AKT ratio in Sertoli cells with and without *IDE* knockdown. Similar results were obtained and showed that *IDE* knockdown increased the p-AKT/AKT ratio, indicating increased AKT activity. Given that aberrant AKT and ERK activation is observed in many human cancers (34-36), we hypothesize that *IDE* deficiency leads to the aberrant activation of AKT and ERK, which promotes Sertoli cell proliferation and results in deficient spermagogenesis in mice and pigs.

In summary, we discovered that reduced *IDE* expression led to enhanced swine Sertoli cell proliferation and increased *WT1* expression. Further analysis of molecular mechanism suggested that the negative regulation of *IDE* expression promoted the activaties of ERK and AKT pathways, which was associated with enhanced cell proliferation. Overall, these findings provide information on the regulatory mechanisms of swine Sertoli cell proliferation and contribute to the future improvement of male reproduction traits in pigs.

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Interest conflict

The authors state no conflict of interest with respect to the research, authorship, and/or publication of this article.

Author's contribution

Conceptualization: Bingyuan Wang; Methodology: Zhiguo Liu, Fei Guo; Formal analysis and investigation: Hongmei Gao, Mingrui Zhang, Yile Sun; Writing: original draft preparation: Bingyuan Wang, Hongmei Gao; Writing: review and editing: Bingyuan Wang; Funding acquisition: Bingyuan Wang, Yulian Mu; Resources: Bingyuan Wang; Supervision: Bingyuan Wang, Yulian Mu.

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